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ORIGINAL PAPER

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Immunity to *Plasmodium berghei* exoerythrocytic forms derived from irradiated sporozoites

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Abstract The nature of immunity generated by *Plasmodium berghei* exoerythrocytic (EE) stages developing from irradiated sporozoites was studied using in vivo parameters of host protection on immunization with irradiated sporozoites and in vitro parameters of inhibition of sporozoite invasion and EE form development by serum antibodies from immunized mice. On in vivo challenge of immunized mice by sporozoites, protection was observed in an irradiation-dose-dependent manner. This finding stresses that protection is dependent on the irradiation dose of sporozoites that allows sporozoite penetration yet controls EE form development within the liver. Using the human hepatoma line Hep G2 as host cells in vitro, we observed that serum antibodies raised in mice immunized with irradiated sporozoites reacted with sporozoite- and hepatic-stage parasites in an immunofluorescent antibody test (IFAT). No reactivity was observed with blood-stage parasites. Serum antibodies from mice immunized with 6- to 18-krad-irradiated sporozoites inhibited sporozoite invasion and caused severe inhibition of EE form development in hepatoma cells, pointing to the antigenic content of EE forms developing from irradiated sporozoites (irra EE forms) as critical immunogens. Moreover, in an enzyme-linked immunosorbent assay (ELISA), serum antibodies raised to 12-krad-irradiated sporozoites showed reactivity to synthetic peptides representing the conserved Region II sequences of the *P. falciparum* circumsporozoite (CS) protein as well as the *P. falciparum* liver-stage-specific antigen (LSA-1)-based repeat sequences, thus implicating an important role for both the sporozoite and the hepatic stage in protection.

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Introduction

Protective immunity to *Plasmodium berghei* can be obtained by vaccination of mice with irradiated sporozoites injected by the intravenous route (Nussenzweig et al. 1967). This immunity has several unique features; it is stage-specific and species-specific but is not strain-specific. Above all it is a very strong, sterile type of immunity that enables the animals to resist very high challenge doses. On the basis of the circumsporozoite (CS) precipitate reaction occurring in the presence of antibodies and the biological effect of monoclonal antibodies directed to the CS protein, it has long been thought that anti-CS antibodies represent the major effector of the protective immunity induced (Ferreira et al. 1987; Nudelman et al. 1989). More recently, however, evidence has been presented that treatment of animals with either anti-CD8+ or anti-interferon-gamma (IFN-γ) antibodies can reverse the protection induced (Schofield et al. 1987). These studies elucidate that multiple effector mechanisms, including antibodies, helper cells, lymphokines, and CD4+ and CD8+ cytotoxic T-lymphocytes, contribute to effective protection. However, these protective mechanisms are compensatory; that is, high levels of antibodies alone can themselves mediate protection in the immunized rodent host (Nardin and Nussenzweig 1993).

A limited degree of protection is conferred by irradiated sporozoites injected by the subcutaneous or intraperitoneal route instead of the intravenous route. Furthermore, it has been shown that irradiated sporozoites are viable organisms that can penetrate the hepatocytes, this being shown both in vitro (Ramsay et al. 1982) and in vivo (Londono et al. 1991). Altogether these data pointed to the idea that protection was dependent upon the ability of a viable sporozoite injected intravenously to penetrate a liver cell and, therefore, to the concept of both the sporozoite and the liver stage of the parasite being targets of the protective mechanisms induced by immunization with irradiated sporozoites. This hypothesis received support from further in vivo/in vitro comparative experiments performed with *P. falciparum* and *P. yoelii,* in

which high and low doses of irradiation were used and in which an in vitro correlate of in vivo observations was made (Mellouk et al. 1990).

However, further investigations were limited by the particular aspects of the biology of the liver stages, i.e., they are difficult to produce in large numbers, and they are short-lived, such that in protection studies the final readout has been only the emergence or lack of emergence of infected red blood cells. It thus becomes clear that a better understanding of those events taking place in the liver cells needs to be acquired to enable the reproduction with a subunit vaccine of the strong type of protection that can be achieved with whole irradiated organisms.

The aim of this work was to identify (a) the effects of sporozoite irradiation at various doses upon protection in vivo, (b) the inhibitory effect of serum antibodies generated by immunization of mice with irradiated sporozoites upon sporozoite invasion and EE form development in vitro, and (c) antigens recognized by serum antibodies from immune mice. Groups of susceptible C57 black mice (Coosemans et al. 1981; Jaffe et al. 1990) were immunized with sporozoites that had been treated with increasing doses of gamma-ray irradiation. Immunized mice were challenged with lethal doses of *P. berghei* sporozoites, and the induced protection was observed. *P. berghei* sporozoite invasion and the development of EE forms originating from sporozoites was studied in vitro in the receptive human hepatoma cell line Hep G2 (Hollingdale et al. 1983).

Materials and methods

Maintenance of the vector and sporozoite production

Breeding of the malaria vector *Anopheles stephensi* was performed in an insectary under standard conditions of 25°C temperature and 85% relative air humidity. Recently emerged adult female mosquitoes were allowed to feed on *Plasmodium berghei* Anka-infected mice. Unfed females and males were removed from the cage after the blood meal. Sporogonic stages of the parasite developed further in these females at 21°C in the following weeks. From day 21 onward, mature sporozoites that had accumulated in the salivary glands were available for experiments.

Irradiation and processing of sporozoites

The dose to the infected mosquitoes was delivered by ^{60}Co irradiation by means of a teletherapy machine (Theratron-780, courtesy Mr. Schaeken, Middelheim Hospital, Antwerp, Belgium). Approximately 50 mosquitoes were placed in an open plastic petri dish covered with a fine cotton cloth. Four petri dishes with mosquitoes were placed side by side within an area of 20 × 20 cm on top of a 1-cm-thick Plexiglass plate and were irradiated with gamma rays from the source placed below. This irradiation setup guarantees a uniform and reproducible dose delivery to the mosquitoes with an overall uncertainty of 2.5%. The irradiation beam was calibrated using an ionization chamber with a calibration factor traceable to primary standards. The dose to the mosquitoes was delivered in the range of 60–180 Gy (1 Gy = 1 J kg^{-1} = 100 rad) and the dose rate was 1 Gy min^{-1}. Mosquitoes were dissected in cold GLSH medium (glucose, lactalbumin, fetal calf serum, hemoglobin; Le Ray 1975), and the salivary glands were transferred to a glass homogenizer on ice and gently disrupted to release sporozoites. The number of sporozoites in the suspension was counted in a hemocytometer.

Animals and immunization protocol

Female 6-week-old C57BL6 mice (IFFA Credo, Brussel) were used for immunizations. Groups of six mice were immunized three times every 2 weeks by intravenous injections of 30,000 irradiated sporozoites of *P. berghei* into the tail vein. Irradiation doses of 6, 8, 10, 12, 15, and 18 krad were selected for the experiments. After each immunization, mice were controlled for blood infection. Sera were collected 7 days after the third immunizing infection. At 2 weeks after the last immunization, mice were challenged with 1,000 sporozoites. From day 4 postchallenge, thin blood films were made from tail blood, fixed with methanol, and Giemsa-stained. The number of parasites per 10,000 erythrocytes were counted and the mean level of parasitemia was calculated. Protected mice were subjected to a rechallenge with 10,000 *P. berghei* sporozoites at 2 weeks after the first challenge.

Culture of EE parasites in Hep G2 cells

Hep G2 cells were maintained in culture as described by François et al. (1991). For parasite culture, 0.81-cm^2 sterile chamber slides (Lab-Tek, Nunc; 8 chambers per slide) were used. On day zero, 50,000 Hep G2 cells were incubated in each chamber in 200 µl of complete MEM Rega 3 medium. On day 4, all medium was removed from the chambers and the monolayers were incubated with 10,000 *P. berghei* sporozoites in 50 µl of MEM Rega 3 medium for 2 h, a period that allows the sporozoites to penetrate into the host cells (Zavala et al. 1985). Uninvasive sporozoites were then removed by replacement of the medium in the chamber slides several times. EE stages were allowed to develop in 200 µl of complete MEM Rega 3 medium for up to 64 h. The medium was not changed during the maturation period.

Assay for quantification of EE parasites

Hep G2 monolayers infected with *P. berghei* sporozoites were incubated for 64 h. Then, cells were fixed with cold methanol and the parasites were counted by means of an immunofluorescent antibody test (IFAT), with the following reagents serving as primary antibodies: (a) an anti-CS protein monoclonal antibody (mAb 3D11 at 50 µg/ml) specific for *P. berghei* (Del Giudice et al. 1987), reactive with the EE stage of *P. berghei* throughout its intracellular development in vitro (Zavala et al. 1985); and (b) serum antibodies generated in mice immunized with irradiated sporozoites (dose 6–18 krad). All parasite numbers given refer to the total number of parasites counted per chamber (0.81 cm^2). Experiments were set up in triplicate and the results were expressed as mean values.

It was necessary to assess the persistence of extracellular sporozoites at the end of the sporozoite – Hep G2 incubation period. An initial experiment was set up to observe if after 2 h of incubation with Hep G2 cells, all the extracellular sporozoites would be removed by medium washes. Hep G2 cells were fixed in situ with 1% glutaraldehyde in a first group of chambers and with methanol in a second group, the difference residing in the cell membrane permeability after fixation. In the first case, only extracellular sporozoites would be available for IFAT labeling; in the second, intracellular as well as extracellular sporozoites would be labeled.

Inhibition of sporozoite invasion

The percentage of reduction (arithmetic mean) in the invasion of *P. berghei* sporozoites (inhibition of sporozoite invasion, ISI) into Hep G2 cell cultures in the presence of serum generated in vivo in

C57BL6 mice after immunization with irradiated sporozoites (dose 6–18 krad) was calculated using the following formula:

$$ISI = [(I_c - I_i)/I_c] \times 100,$$

where I_c represents the number of sporozoites that invaded in the presence of normal C57Bl6 mouse serum (this serum was collected from naive unimmunized mice at the same time as were the sera from sporozoite-immunized mice and was stored under similar conditions) and I_i represents the number of sporozoites that invaded in the presence of immunized mouse serum.

ISI activity was measured by incubating 10,000 sporozoites suspended in 50 µl of MEM Rega 3 medium together with 50 µl of heat-denatured serum diluted 1/40 in phosphate-buffered saline (PBS) in chambers containing Hep G2 monolayers. The final dilution of serum in the chambers was 1/80. After 2 h, all medium was removed from the chambers, and after several medium washes with fresh MEM Rega 3 the intracellular sporozoites were counted after IFAT staining using the 3D11 mAb.

Inhibition of EE development

Immunized mice serum were also assayed for the inhibition of EE form development in Hep G2 cells. Sporozoite invasion was allowed to proceed for 2 h. Thereafter, 50 µl of either immunized mouse serum or normal mouse serum collected at the same time and stored under similar conditions was diluted to a final ratio of 1/80 in complete MEM Rega 3 medium and then added to Hep G2 monolayers after removal of the sporozoite suspension. After incubation for 64 h an IFAT with mAb 3D11 was done to count the number of EE forms that had developed in the presence of immunized mouse sera. The percentage of inhibition of EE development was calculated using the following formula:

$$\% \text{ EE form inhibition} = [(E_c - E_i)/E_c] \times 100,$$

where E_c represents the EE forms developing in the presence of normal mouse serum and E_i represents the EE forms developing (at 64 h) in the presence of immunized mouse serum.

Enzyme-linked immunosorbent assay

Sera raised in mice immunized with 12-krad-irradiated sporozoites were assayed in an ELISA for the presence of antibodies to synthetic peptides representing the conserved Region II sequence of *P. falciparum* CS protein (345–362; EWSPCSVTCGNGIQVRIK) and the *P. falciparum* LSA-1-based repeat hybrid sequence (EQQSDLEQERLAKEKLQEQQSDLEQERRAKEKLQ). Briefly, wells of a flat-bottomed microtiter plate (Greiner, Nuringen, Germany) were coated with 2.5 µg of peptide in a 50-µl volume of Na_2CO_3-$NaHCO_3$ coating buffer (pH 9.6). Separate wells were coated with 2.5 µg of a synthetic peptide representing the *P. falciparum* CS protein-based T cell epitope, CST3, whose sequence (DIEKKIAKMEKASSVFNVVNS; 378–398) has been reported to be a conserved and universal T-cell epitope (Sinigaglia et al. 1990). Separate wells were coated with a *P. berghei* spor0ozoite lysate. The lysate was prepared by repeated freeze-thawing of a sporozoite suspension in GLSH. After further dilution in coating buffer a final volume of 50 µl of lysate, corresponding to about 700 sporozoites, was dispensed in each microtiter well.

Plates were incubated until dry and the uncoated reactive sites in the wells were blocked by incubation with PBS containing 5% nonfat milk powder. After incubation and washing, 50 µl of a 1/100 dilution of serum was added to each well. Plates were further incubated, and then 50 µl of a goat anti-mouse antibody conjugated to horse-radish peroxidase was added to each well at a 1/1,000 dilution, followed by 100 µl of a substrate solution containing *o*-phenylenediamine dihydrochloride. The reaction was stopped with 8 N sulfuric acid, and the optical density (OD) of the reaction product was measured with an ELISA reader at 490 nm. Results were obtained as the mean of triplicate values. Negative control values were obtained by incubating normal mouse serum at a 1/100 dilution in peptide-coated wells.

Results

Immunization with irradiated sporozoites

In a series of experiments, groups of 6 C57BL6 mice received 3 intravenous immunizing injections of 30,000 sporozoites each at 14-day intervals. Sporozoites were irradiated with different doses of gamma rays ranging from 6 to 18 krads. In the group of mice immunized with 6-krad-irradiated sporozoites, two of the six mice developed blood parasitemia after immunizing infections and died, indicating that at least some sporozoites remained virulent. At an 8-krad or higher radiation dose, no blood infection occurred, indicating that all sporozoites were attenuated.

Challenge of immunized mice

All groups of immunized mice were subjected to 2 successive challenges with 1,000 and 10,000 live sporozoites, respectively. A challenge with 1,000 sporozoites (Fig. 1a) induced in all the unimmunized control mice a blood parasitemia that was detectable on day 4 and was lethal. In mice immunized with irradiated sporozoites, complete protection (a situation where none of the challenged mice develops blood parasites) was observed in the groups immunized with 12- and 8-krad-irradiated sporozoites. Partial protection was observed in the group of mice immunized with 15- and 10-krad-irradiated sporozoites; four to five of the six immunized mice in these groups were protected against challenge. Two of the six mice immunized with 18- and 6-krad-irradiated sporozoites were also protected.

However, when protected mice in each group were rechallenged with 10,000 live sporozoites, a complete protection profile was obtained only in the group of mice immunized with 12-krad-irradiated sporozoites (Fig. 1b). Few of the mice (one or two) in the other groups were protected from infection. These results were reproducible in successive experiments.

In vitro activity of sera from immunized mice

At 1 week after the last immunization, serum were collected from each group of immunized mice and were assayed by IFAT for binding to sporozoites, mature EE forms, and blood forms (Table 1). Antiserum raised in mice immunized with irradiated sporozoites contained antibodies directed to both sporozoites and mature (64-h-old) EE forms (up to dilutions of 1/160) but not to blood-stage antigens. However, lower titers were obtained with serum from groups of mice immunized with 6- and 8-krad irradiated sporozoites.

Sera from mice immunized with sporozoites irradiated at various doses were also assayed for ISI into Hep G2 cells in vitro and for the inhibition of EE form development in these cells (Table 1). An initial experiment

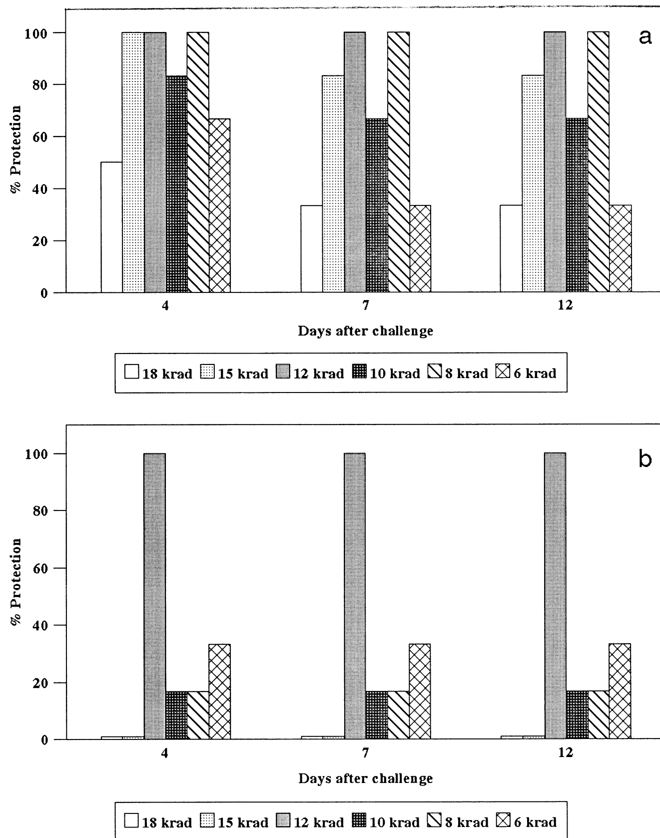

Fig. 1a, b Protection obtained by immunization of C57 black mice with irradiated sporozoites. **a** Challenge with 1,000 sporozoites. **b** Rechallenge with 10,000 sporozoites

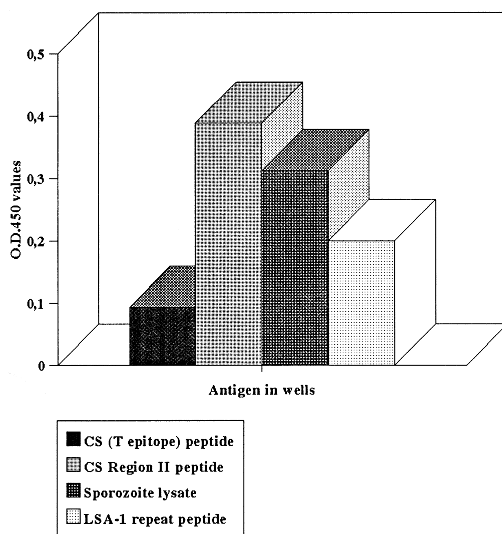

Fig. 2 Reactivity of *Plasmodium berghei* anti-sporozoite sera with *P. falciparum CS* and LSA-1-based peptides and with *P. berghei* sporozoite lysate

Table 1 ISI activity and EE form reduction in vitro, by serum generated in mice immunized with irradiated sporozoites.

Immunization with sporozoites irradiated at	Serum inhibitory activity (%) on		IFAT titers upon		
	Invasion	EE dev.	SPOR	EE	BS
6 krad	5	52	1/80	1/80	–
6 krad	5	52	1/80	1/80	–
8 krad	3	55	1/80	1/80	–
10 krad	11	68	1/160	1/160	–
12 krad	16	77	1/160	1/160	–
15 krad	11	80	1/160	1/160	–
18 krad	27	78	1/160	1/160	–

[a] Binding to sporozoites (SPOR), EE parasites, and blood-stage parasites (BS) was observed by IFAT using the respective serum as the primary antibody

was done to assess the persistence of extracellular sporozoites at the end of the sporozoite-Hep G2 incubation period, which may interfere with the correct counting of intracellular sporozoites. Sporozoite incubation was followed by repeated changing of the medium in the chambers. In the chambers containing glutaraldehyde-fixed cells, an average of 41 ± 14 sporozoites/chamber (0.81 cm^2) were counted, which were probably adhering to the Hep G2 cell surface. In the absence of glutaraldehyde fixation, an average of 1518 ± 86 sporozoites were counted in each chamber, the sharp difference being due to the counting of intracellular sporozoites. It may be concluded that most of the extracellular sporozoites are removed by medium changes such that a large proportion of the parasites demonstrated by IFAT staining after methanol fixation are intracellular.

The percentage of ISI activity provided by antiserum raised in mice immunized with 18-krad-irradiated sporozoites was 27%. Sera from mice immunized with 6- to 15-krad-irradiated sporozoites provided lower levels of blocking antibodies, i.e., 5–11% ISI activity.

The inhibition of EE form development was pronounced. Antiserum from mice immunized with 12- to 18-krad-irradiated sporozoites provided EE form inhibition of almost 80% as seen at 64 h of development in Hep G2 cells.

Plasmodium berghei anti-sporozoite antibodies cross-react with *P. falciparum* peptides

Serum antibodies generated in mice immunized with 12-krad-irradiated sporozoites of *P. berghei* showed reactivity in ELISA with the sporozoite lysate as well as the peptides based on the *P. falciparum* Region II sequence of CS protein and the LSA-1 repeat sequence (Fig. 2). Significant reactivity with these peptides was observed at a serum dilution of 1/100 as compared with the OD_{450} values obtained for wells coated with the CS-based T-epitope peptide. Results depicted as the mean values of triplicate wells suggested that serum antibodies recognized epitopes on CS Region II- and LSA-1 repeat-based peptides as well as on the native sporozoite protein and portrayed the cross-reac-

tivity of serum antibodies with *P. falciparum* antigens. As a negative control, respective peptide-coated wells were also incubated with normal mouse serum (1/100 dilution), and the OD_{450} values in these wells did not exceed 0.02.

Discussion

Triggering of protective mechanisms depends on hepatic development of the parasite

Within the scope of the experimental rodent model of *Plasmodium berghei*, the C57BL6 mouse strain is extremely susceptible to *P. berghei* infections as compared with the BALB/c strain. We decided to work with the C57BL6 strain of mice with the aim of establishing some of the parameters necessary to obtain protection by *P. berghei* irradiated sporozoite immunization, since in so doing we may be studying parameters necessary to confer solid protection.

Previous studies suggest that the schedule of sporozoite immunization as well as the irradiation dose of sporozoites are indeed critical factors in the establishment of protection to challenge infections. In our immunization studies, only multiple immunizations were effective in triggering protection against sporozoite challenge. The same dose given in a single immunization is incapable of conferring host protection. For a susceptible host such as the C57BL6 mouse the requirement of booster immunizations for triggering protective immunity could stress the importance of appropriate stimulation of the host memory cells during effective immunization procedures.

Our results suggest that it may be not only the antigens expressed by the sporozoite stages but also those expressed by the hepatic forms that are triggering protective immunity. Sporozoites irradiated at 18 krad enter Hep G2 cells in vitro (data not shown), yet the subsequent EE forms perish soon, and this immunization regimen gives poor protection in vivo. Reports claim that sporozoites exposed to high doses of irradiation are no longer effective immunogens, probably since liver-stage antigen expression does not occur. In our experiments, mice immunized with 12-krad-irradiated sporozoites were completely protected against a challenge with 10,000 *P. berghei* sporozoites. At this irradiation dose, sporozoites develop into young trophozoites in Hep G2 cells in vitro and persist for several days in the hepatoma cells as immature forms (data not shown), thereby suggesting these stages to be the critical immunogens required for protection. Considering the high susceptibility of C57BL6 mice to *P. berghei* infections, protection against challenge with 10,000 sporozoites indicates that key protective mechanisms that may themselves depend on the expression of novel epitopes are generated in vivo upon immunization with 12-krad-irradiated sporozoites. This study also confirms previous speculations that changes in the protection mediated by irradiated sporozoites can be brought about by variations in the irradiation dose, which themselves influence the outcome of the parasite in liver cells.

Polyclonal blood-stage serum, which labels mature EE parasites (Suhrbier et al. 1989), recognizes antigens in EE forms obtained from sporozoites irradiated at less than 10 krad, but not when the latter have been irradiated at a 12-krad or higher dose (data not shown). The 12-krad-irradiated EE forms do not develop to 48-h-old "normal" EE forms in vitro, as shown by the lack of detectable blood-stage antigens, but persist as immature forms within the Hep G2 cells. Thus, variations in the sporozoite irradiation dose lead to either an incomplete faulty development or some further degree of maturation of the irradiated EE forms. In this manner, sporozoite irradiation doses regulate the expression or lack of expression of protective epitopes by the developing EE parasites resulting from irradiated sporozoites. This might be taken as indicative that there would be a differential expression of particular liver-stage antigens between high- and intermediate-dose irradiation (i.e.,18 and 12 krad) that would be critical in inducing protection and confirms that transformation of sporozoites into young liver stages is an obligatory step in the induction of strong protection.

Mediators of immune protection

In the rodent model the assays thought to correlate with protection by antibodies from sporozoite challenge are the circumsporozoite precipitation (CSP) assay, the ISI assay, and, more recently, the inhibition of exoerythrocytic form (EE) development assay. The effect of antibodies, particularly the biological effect of mAbs, has influenced the research on a sporozoite vaccine. However, often the effects of mAbs could not be reproduced by the polyclonal response to the corresponding antigen. Passive transfer of mAbs to *P. berghei* CS protein (Potocnjak et al. 1980) and mouse anti-*P. berghei* CS peptide antibodies (Egan et al. 1987) have partially protected mice against sporozoite challenge, and it is possible that the failure of passively transferred anti-sporozoite antibodies may reflect low concentrations rather than the absence of activity.

It is noteworthy that serum from mice immunized with irradiated sporozoites were capable of exerting a strong inhibitory effect upon EE development and/or survival in vitro in Hep G2 cells (up to 80% inhibition of EE form development). An assay conducted to detect IFN-γ (interferon gamma) in these immunized mice serum showed that no detectable level of this cytokine could be detected, suggesting that the inhibitory activity upon EE forms is antibody-mediated. It is possible that on entering the parasitophorous vacuole (PV), serum antibodies may not only prevent the development of trophozoites but even destroy them. Recombinant and synthetic peptides based on the repeat sequences of *P. falciparum* CS protein generate antibodies upon the immunization of mice that inhibit schizont development (Mazier

et al. 1986). In the presence of mAb NYS1 directed to the *P. yoelii* CS protein, not only do the trophozoites fail to develop into schizonts in primary hepatocyte cultures, but by 48 h postinvasion, most parasites have disappeared as compared with control cultures (Nudelman et al. 1989). The proposed explanation given for the continuous destruction of the hepatic parasites by the latter authors was that anti-CS antibodies bound to the parasite at the time of sporozoite invasion and blocked the translocation of the CS protein into the parasitophorous vacuole (PV), leading to acidification of the vacuole. This decrease in the pH within the PV led to lysosomal digestion of the trophozoite.

In our experiments, serum antibodies from mice immunized with irradiated sporozoites had a more inhibitory effect on EE form development than on sporozoite invasion in vitro, indicating that serum antibodies may be directed not only to antigens expressed by the CS protein but also to those expressed by the hepatic stages. In our experiments conducted to look for EE form inhibition by immunized mouse sera, serum antibodies were added to Hep G2 cultures after sporozoite invasion had been completed. Future experiments will check for the inhibitory effect of these sera on infected hepatocyte cultures. Such an effect of antibodies upon EE form development has seldom been explored in the past and would deserve further study.

The antigenic repertoire of the EE parasites developing from irradiated sporozoites includes some of the sporozoite antigens, such as the CS protein, as well as antigens that are unique to the EE forms, such as the LSA-1 antigen (Guerin Marchand et al. 1987) and other liver-stage (LS) antigen or antigens shared by liver and sporozoite stages (Druilhe and Marchand 1989; Hollingdale et al. 1990a, b). Sera from mice immunized with 6- to 18-krad-irradiated sporozoites contained antibodies to both the sporozoite and EE stages. Indeed, serum antibodies from 12-krad sporozoite immunized mice have shown specific reactivity with synthetic peptides based on *P. falciparum* CS protein and LSA-1-based epitopes. Conserved regions on invariant malaria antigens may contain crucial B-cell epitopes, and such peptide sequences from the human malaria parasite, *P. falciparum*, can be screened in the *P. berghei* experimental animal model.

Until recently the liver stages of the life cycle of the malaria parasite have received little attention due to the difficulty of obtaining liver-stage parasites and also to the general belief that the liver stages were immunologically "silent." Recent evidence, however, proves that immunological mechanisms can act upon these stages. Taken together, our results clearly suggest that in the so-called *sporozoite* vaccine, it may be not only the antigens expressed by sporozoites but also those expressed when the parasite lies within the hepatocyte that are essential in the induction of protection.

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